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In vitro Studies on the Antiviral Effect of Olive Leaf Against Infectious Laryngotracheitis Virus

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Abstract: A commercial plant extract derived from olive tree leaf (*Olea europaea*) (LExt), was found to inhibit the *in-vitro* infectivity of Infectious laryngotracheitis Virus (ILTV); this virus belongs to subfamily alpha herpesviridae. Incubation of virus with LExt before infection reduced the viral infectivity. Furthermore, LExt drastically decreased ILTV titers in a dose dependent manner when added to chicken embryo rough cell 36 hrs post-infection. On the other hand, the LExt was able to inhibit cell-to-cell membrane fusion induced by ILTV in uninfected cells, suggesting interactions with viral envelope. Therefore, on the light of this finding, it was propose that O. europaea could be used as a promising natural potential antiviral agent.

Key words: Olive leaf extract · ILTV · antiviral · viral membrane fusion

INTRODUCTION

Many screening efforts have been made to find antiviral agents from natural sources. Plants have long been used as remedies and many are now being collected and examined in an attempt to identify possible sources of antivirals [1]. Antivirals have been used to control infections caused by viruses [1-5]. Leaves from olive tree (*Olea europaea*) are found to be rich in biophenols (BPs; Fig. 1), such as Oleuropein (Ole), verbascoside, ligstroside, tyrosol or hydroxytyrosol. Some of these compounds have demonstrated antimicrobial activity by inhibiting the growth of a wide variety of bacteria, fungi and viruses [6, 7]. Recently, in a U.S. patent, LExt has been claimed to have potent antiviral activities against Herpes hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus and feline leukemia virus [8]. In addition, LExt has also exhibited a significant antiviral activity against respiratory syncytial virus and parainfluenza type 3 virus [9]. Up till now, the mechanism of its antiviral activity is unknown.

Infectious laryngotracheitis is one of the most serious respiratory diseases affecting poultry industry in Egypt [10]. The survived cases failed to gain the presumed body weight in case of meat production and showed drastic decrease in egg production during the course of the disease in case of egg producing flocks, whereas the disease infect poultry at any age. The signs of the disease are usually acute with high flock morbidity.

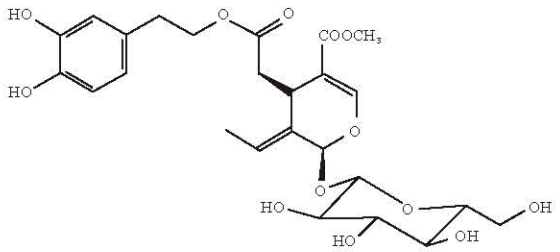

Fig. 1: Chemical structure of Oleuropein, the major component of the olive

The morbidity of almost 100% and mortality ranging from 0 to 49%.Gasping follows nasal discharge, lacrimation and moist rales; in the later stages dyspnea occurs with extension of the head and neck. In acute cases and depending on the virulence of the virus, expectoration of bloody mucus may occur. In some affected flocks, this sign was so prevalent that the walls and equipment were spattered with dried blood [11, 12]. The causative agent of ILT is a virus belongs to subfamily alpha herpesvirinae and initially propagated in the Chorio-allantoic Membrane (CAM) of developing chicken embryos. With the advent of cell-culture techniques, researchers quickly established that ILTV would replicate in adult chicken kidney cells as well as in a variety of chicken embryo-epithelial cells such as kidney, liver and lung [13]. The virus was successfully propagated on Chicken Embryo Rough CER cells [14]. The aim of the present study is to investigate the antiviral activity of olive extract on ILT virus.

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MATERIALS AND METHODS

Olive leaf powered extract (Lext): It was purchased as dried leaves and then crushed and a water extract was made of it in different concentrations such as 0.2, 0.4, 0.6, 0.8 and 1 mg ml^{-1}.

Chicken Embryo Rough cells (CER) and virus: CER cells were purchased from VACSERA, Agoza, Egypt while the virus was available at our laboratory [14].

Plaque assay: The virus was added in different concentrations on confluent layers of CER cells grown on 24-well plate. One hour after virus adsorption at 37°C, the medium was replaced by 0.5ml of 0.75%methylcellulose (Sigma, ST. Louis, MO, USA) in RPMI-1640 cell culture medium supplemented with 2% of FCS. Plates were incubated at 37°C until plaque formation (3-4 days). After fixing and staining with a 0.1% crystal violet solution in formalin [15], the number of plaques was counted by eye inspection. The result was expressed as plaque forming units (pfu).

Virus purification: It was prepared according [15] using sucrose gradient ultracentrifugation. Where the confluent CER cells were infected by10^3 pfu of ILT virus per cell. After 72 hrs post infection, cells were frozen and thawed three times and centrifuged at 5000 rpm for 15 min and the supernatant was collected and stored at -20°C. The previously prepared supernatant was placed in Beckman TL-100 ultracentrifuge plastic centrifuge tube and exposed to high speed centrifugation at 45.000 Xg (100.000 rpm) for 2 hrs to pellet the virus on the bottom of the tube. The virus pellet was resuspended in this buffer which contained 20 mM sodium chloride and different concentration of sucrose gradient 5 to 45% then centrifuged at 45.000 Xg (100.000 rpm) for two hrs the white band of the purified virus was sucked through sterile Pasteur pipette and stored in 2 ml epindroff tube at-70°C until used. After performing the plaque assay, the virus was concentrated to 10^{11} pfu [14].

Cytotoxicity assays: The Cytotoxicity effect of LExt on CER cells by quantifying the CER cells viability using MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-teyrazolium] (Sigma, ST. Louis, MO, USA). Cytotoxicity was examined after 2 days (to examine acute Cytotoxicity) and after 7 days (to examine chronic Cytotoxicity) of CER cells exposure to different concentrations (from 1 to 1450 μg ml^{-1}) of LExt. Cell cytotoxic dose 50 (CC_{50}) values, defined as the concentration at which a 50% cytotoxic effect was observed after 7 days of cell treatment were calculated as the compound concentration required to reduce the MTS signal to 50% of the untreated CER cells [16].

Virus titration: To test the influence of pre-incubation of ILT virus with *O. europaea* on ILTV infectivity, a previously developed immuno-staining plaque assay was used [17-19]. Briefly, LExt at different concentrations (up to 54 μg/ml) were incubated with ILTV during 2 hrs at 37°C in RPMI-1640 cell culture medium supplemented with 2% foetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM l-glutamine, 500 μg ml^{-1} gentamicin and 25 μg/ml amphotericin B. After incubation, the mixtures containing ILTV (200 pfu/well) plus compounds were added to CER cells, grown in 96 well plates, during 2 h at 37°C. Alternatively, cells, which had been pre-incubated with the same concentrations of LExt, during 2 h at 37°C, were infected with ILTV (200 pfu/well). Then, in both cases the infected CER cells were washed, medium devoid of inhibitors added and the plates incubated during 24 h at 37°C. After incubation (24 hrs), the CER cells were fixed during 10 min in cold methanol and air-dried. Hyper immune serum against ILTV provided by our lab [14] diluted 1000-fold in dilution buffer (0.24 mM merthiolate, 5 μl Tween 20, 50 mg/l of phenol red in PBS pH (6.8) were added to the wells (100 μl/well) and incubated during 1 h at room temperature. After washing with distilled water, 100 μl of peroxidase-labelled rabbit anti-IgG mouse antibody (Ab) (Sigma, ST. Louis, MO, USA) were added per well and incubation was continued during 30 min. After three washings by immersion in distilled water, 50 μl of 1 mg ml^{-1} per well of Diamino Benzidine (DAB) (Sigma, St. Louis, MO, USA) in PBS containing H_2O_2 were added [20, 21] and the reaction allowed to proceed until brown foci were detected with an inverted microscope. Once washed with water and air dried, brown foci (DAB-stained foci) of 15-20 brown or DAB-stained cells (DAB-stained single cells) were counted with an inverted microscope (Leica Ltd., Cambridge, UK) with a 10× ocular eye grid [22]. The results were expressed as the percentage of infectivity and calculated by the formula: (number of plaque in the presence of compounds/total number of plaque in the absence of compounds) ×100. The concentration at which an infectivity inhibition of 50% was observed, 50% inhibition concentration (IC50), was defined as the concentration of compounds,

which reduced the percentage of ILTV-induced plaque by 50% with respect to untreated virus and expressed in µg/ml.

Fusion induced by addition of ILTV to uninfected CER cell cultures. The low pH (pH 6) ILTV-induced cell-to-cell fusion in uninfected EPC cell cultures was estimated by a syncytia formation assay [19]. ILTV (8×10^9 pfu/ml) was incubated with media, LExt at 54 µg/ml in RPMI-1640 supplemented with 20 mM HEPES, 20 mM MES and 2% FCS at pH 7.4for 2 h at 37oC. ILTV (8×10^6 pfu/well), ILTV+ LExt (8×10^6 pfu/well), LExt at 54 µg/ml was added to CER cells 96-well plates in a final volume of 100µl per well. The plaque assay was performed as previously described [19]. To measure the extent of fusion, cells were then fixed with cold methanol, dried and stained with Giemsa (5 mg ml^{-1} in phosphate saline buffer) and number of nuclei in syncytia of three or more nuclei per syncytia was counted among10,000 nuclei per well. The percentage of fusion was calculated by the formula, number of nuclei in syncytia/total number of nuclei×100 [20]. The results were expressed as the percentage of fusion relative to the one induced by ILTV by the following formula, percentage of fusion induced by ILTV pre-incubated with Lext/percentage of fusion induced by ILTV×100 [19]. Alternatively, ILTV was added to CER cells that had been pre-incubated with the same concentrations of LExt and after washing cells, fusion was induced as above.

RESULTS

Cytotoxicity of Lext: To explore the potential use of LExt as an antiviral agent, firstly, its possible *in vitro* acute and chronic toxicity was tested. LExt showed no cytotoxic effects on CER cell cultures at concentrations lower than 1 mg ml^{-1}. The cytotoxic concentration 50% values (CC50), i.e. the concentration corresponding to a 50% cytotoxic effect after 7 days of cell treatment, for LExt was of 1250 µg/ml.

Inhibition of ILTV infectivity by Lext: Plaque assay was first performed to calculate pfu in each test to be used (Photo 1.) The inhibitory effect of LExt on ILTV infectivity, measured as the number of plaque formation in CER cells, is shown in Fig. 2. The LExt inhibited plaque formation of ILTV in a dose dependent manner when

Photo 1: Plaque Assay on the right normal CER cell control and in the left CER cell 72 hours post infection

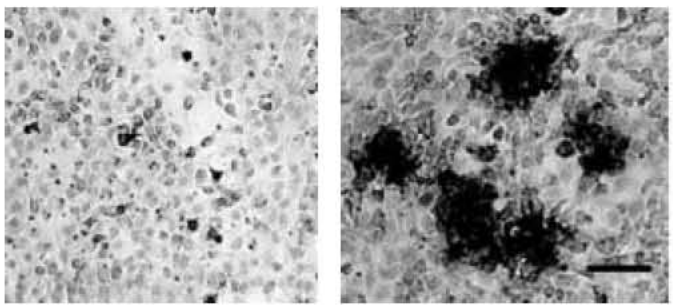

Photo 2: Inhibition of ILTV infectivity by LExt. Plaque of infected cells from CER cell cultures infected with ILTV (B) and ILTV+ 54 µ/ml LExt (A)

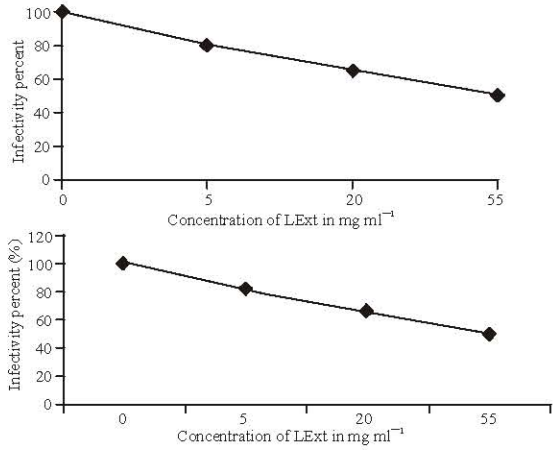

Fig. 2: Inhibition of ILTV infectivity by LExt. On the right ILTV was pre incubated with LExt at indicated concentrations prior to infection of CER cells. After infection time CER cells were washed. On the left CER cells were incubated with LExt, washed and then infected with ILTV

pre-incubated with ILTV before the infection (Fig. 2). Pre-incubation of ILTV with a concentration as low as 2 μg/ml of LExt reduced ILTV infectivity to about 65% of the untreated ILTV controls. The percentage of infectivity decreased steadily as the compound concentrations were increased up to a minimum value of ~10% at54μg/ml of LExt. This reduction of the viral infectivity is clearly observed in the pictures showed in Fig. 2 where the infection plaque were drastically reduced at 54 μg/ml LExt (Photo 2.A) and almost completely abolished at 54 μg/ml LExt (Photo 2.B). The 50% inhibitory concentration, IC_{50} value, which is the concentration of compounds required inhibiting ILTV infectivity to 50%, was calculated for Lext =5.89 μg/ml. In addition, when the CER cells were pre-incubated with LExt at a concentration of 54 μg/ml (much lower than the concentration needed for cytotoxic effects) and then infected with intact virus, the ILTV infection was also reduced to 62% (Fig. 2).

ILTV titer decreases in ILTV-infected CER cells by post-infection treatment with Lext: In order to get some insight into the mechanism of action of LExt, the LExt was investigated whether could reduce ILTV titers when added after several rounds of ILTV replication in CER cells. For that, CER cells were infected with ILTV and 36 hrs later, LExt was added to the infected cell cultures (no compounds were added to control cultures). Two days after the addition of the compound (3.5 days post-infection), ILTV titers were determined in the ILTV-

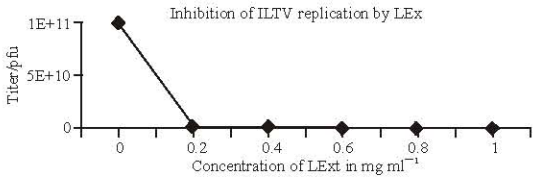

Fig. 3: Lext inhibits ILTV replication when added to ILTV-infected CERcells. At 0 h and 36 hrs post-infection, LExt at the indicated concentrations was added to ILTV infected-CER cells. Two days later, supernatants of infected cultures were harvested and the titer of recovered ILTV was estimated by the plaque assay.

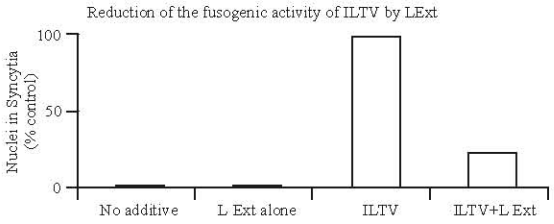

Fig. 4: Fusogenic activity of ILTV is reduced by pre-incubation of virus with LExt. Cell-to-cell fusion of uninfected CER cells was induced by adding untreated ILTV, ILTV pre-incubated with LExt (ILTV + LExt), LExt to CER cells. Fusion induced by untreated ILTV was considered as 100% value

infected cell culture supernatants by a plaque reduction assay. Fig. 3 shows the virus yield obtained in presence of 0.6, 0.8 and 1 mg ml^{-1} of LExt. In this experiment LExt concentrations were at least 1×log higher than in Fig. 2 because now they are used to inhibit the higher viral loads resulting from a productive infection. In untreated ILTV infected CER cell cultures (control cultures), ILTV replicated normally and the viral titer recovered from supernatants of infected cultures was >4×10^9 pfu/ml. In contrast, the viral titer recovered from infected CER cells treated 36 hrs post infection with LExt was significantly lower and decreased as the concentration of LExt was increased in a dose dependent manner (Fig. 3). At a concentration of 0.8 mg ml^{-1}, LExt almost completely inhibited the progression of the infection (less than 100 pfu/ml in culture supernatant) and at 1 mg ml^{-1} no virus was recovered (Fig. 3). However, we can not rule out that the lower titer could be due to some toxic effect of LExt at this concentration.

ILTV-induced plaque in CER cells is inhibited when ILTV is pre-incubated with Lext: Fusion of viral and cell membranes is a step in alpha herpes virus infection. To

determine the influence of LExt on membrane fusion process, fusion was induced in cultures with ILTV treated or not treated with the LExt. When ILTV was pre-incubated with LExt-before its addition to the CER cells, the percentage of induced fusion dropped from 100 to 22% of the control. LExt did not induce significant percentages of fusion in the absence of ILTV (Fig. 4). In contrast, when fusion was induced with ILTV in CER cells, previously treated with the same concentrations of the LExt, the fusion percentage values were similar to the controls. This fact indicates that LExt interacts with the viral envelope producing ILTV particles with a reduced membrane-fusion capacity.

DISCUSSION

Natural products are a relevant source of antiviral drugs [1, 23]. For example, antiviral activities of phytochemicals have been described for vesicular stomatitis virus VSV [24-26]. Olive has been shown to possess a potent antiviral activity against other DNA and RNA viruses [8, 9]. Due to the above-mentioned facts and in order to search for safe and effective natural antiviral agents against ILTV, the effect of LExt was examined on ILTV infectivity. The cytotoxic concentration 50% values (CC_{50}) after 7 days of cell treatment for LExt was of 1250 μg/ml, Similar CC50 values for LExt have been found in other different mammalian cell lines [23]. The results presented here show the inhibitory action of LExt against ILTV when the virus was incubated with the agents before infecting the cells (Fig. 2A), suggesting a direct inactivation effect of the ILTV infectivity by this compound. However, pretreatment of the CER cells with LExt prior to infection with untreated virus also inhibited infection although this effect was only partially (Fig. 2). These results suggest that the compounds may interact with or persist locally on the EPC cell surfaces. In addition, the LExt could effectively reduce the progression of ILTV infection when added after several rounds of ILTV replication (Fig. 3). Concretely, LExt was able to abolish virus titer (Fig. 3) in CER cells infected with ILTV when added 36 hrs after cell infection. The therapeutic index (TI =CC_{50}/IC_{50}) found for LExt in this study was approximately 255, which indicates a higher *in vitro* antiviral activity than those values previously published for LExt [21].

Many studies have shown that Olive possesses a strong anti-oxidative activity [27, 28] but this property does not seem directly related to its antiviral effect [9]. On the other hand, the interaction of LExt with the surface of phospholipids bilayers has previously been reported [29]. When ILTV was pre-incubated with the LExt, cell-to-cell fusion induced at low pH by ILTV in uninfected cells was reduced to 25-30% (Fig. 4), therefore an effect of the LExt on lipid/protein components present on the ILTV envelope and involved in membrane fusion could be possible [30]. It has previously been published that the interaction of the G glycoprotein of some DNA viruses with anionic phospholipid components of cellular membranes seems to be a necessary step for a successful viral fusion [31-34]. In this way, LExt might induce changes on the ILTV envelope, which could interfere the interaction of glycoprotein G to anionic phospholipids domains of membranes and therefore inhibit the early fusion steps.

Therefore, on the light of this finding, it was propose that *O. europaea* could be used as a promising natural potential antiviral agent for ILTV.

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